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The goal of this research is to study the association of the estrogen receptor with the nuclear matrix, and to identify the nuclear matrix acceptor sites for the estrogen receptor. We have constructed an ER fusion protein (GFP-ER) with a His_6 tag, HA tag, and the green fluorescent protein. In transiently transfected ER negative cells GFP-ER causes the ligand dependent transactivation of an ERE-reporter construct. Using confocal fluorescent microscopy we observed the cellular localization of GFP-ER in living cells. GFP-ER is localized to the nuclei, with exclusion from the nucleoli, both in absence of ligand, and when 17β-estradiol, or 4-hydroxytamoxifen are added. In MCF7 cells (ER+), the nuclear distribution has a speckled or punctate pattern which does not change with the addition of ligand. In MDA MB 231 cells (ER-), the distribution of GFP-ER in cells without ligand added is fuzzy or diffuse, with the addition of ligand the nuclear distribution becomes more localized or granular. The pattern of distribution of GFP-ER appears the same on nuclear matrices isolated on slides as in living cells. We corroborated this by extracting nuclear matrix in solution and detecting the presence of GFP-ER by Western blotting with HA-antibody.

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INTRODUCTION

A. Rationale

At presentation only 30 to 40 percent of human breast cancers are hormonally dependent, that is, they will respond to some form of endocrine manipulative therapy, e.g., antiestrogen therapy. However, most human breast cancers originate as hormonally dependent tumours as ovariectomized, non-estrogenized woman (Turner's Syndrome) and males rarely get breast cancer [1]. While the mechanisms responsible for hormone dependence in human breast cancer are complex and mostly unresolved, the hormonally-dependent phenotype correlates strongly with the presence of estrogen receptors (ER) in the tumor [2]. Thus, about 60% of ER+ human breast tumours respond to antiestrogen or other endocrine therapies [3]. However, most of these tumours will eventually develop resistance to endocrine therapies, despite continued ER expression.

ER is a nuclear matrix bound transcription factor that binds to nuclear matrix proteins (acceptors) [4-8]. There is evidence that the ER acceptors differ in antiestrogen-sensitive and -resistant breast cancer cells [8]. Identification of these proteins may be informative in revealing whether an ER+ breast cancer is sensitive to antiestrogen therapy. We have designed a novel strategy to isolate and characterize the nuclear matrix acceptors for ER.

B. Nuclear Matrix and Transcription Domains

The nucleus is highly organized, with transcribed genes being found in discrete foci [9-14]. Transcribed and nontranscribed sequences are precisely compartmentalized within the nucleus, with transcriptionally active chromatin being selectively bound to the nuclear matrix [15-19]. The nuclear matrix is a three-dimensional nuclear network that provides the structural support for enzymes and proteins (e.g., transcription factors) involved in nuclear processes such as transcription, replication and DNA processing. The nuclear matrix, a DNA-protein structure, is visualized following removal of chromatin from nuclease digested nuclei [20-23] (Fig. 1). Actively transcribed chromatin regions are attached to the nuclear matrix by multiple dynamic attachment sites [16] (Fig. 2). The transcription machinery, specific transcription factors (e.g., ER), and nuclear enzymes are thought to mediate the dynamic attachments between transcriptionally active chromatin and nuclear matrix [24-30].

The nuclear matrix is selective about which transcription factors it binds, and this selectivity varies with cell type [25,31,32]. It has been postulated that the nuclear matrix has a role in the expression of genes by concentrating a subset of transcription factors at specific nuclear sites [28,33]. Transcription factors associated with the nuclear matrix include ER, YY1, AML-1, Sp1, Oct1, mutant p53, and Rb [25,28,31,34-40].

C. Estrogen Receptor and Nuclear Matrix

The first step in the mechanism by which estrogens stimulate growth and antiestrogens inhibit growth of many ER+ human breast cancer cells is the direct binding of ligand to ER [41]. Unliganded ER can also be activated indirectly by phosphorylation, a process involving the MAP kinase signal transduction pathway [42], or directly by binding to cyclin D1 [43]. Steroid hormone receptors are DNA-binding proteins that can transcriptionally regulate target genes by directly interacting with the gene's promoter elements [44,45]. A consensus estrogen response element (ERE) consisting of a palindromic DNA sequence that binds ER as a monomer or dimer has been identified [44,45]. ER can interact synergistically with several transcription factors and bind to a half palindromic ERE [44-51]. Studies show that hormone receptors can activate transcription without binding to DNA [52,53]. This indirect regulation appears to require other *trans*-acting factors to mediate the hormone response. Recently, a novel ER, ERβ, which is expressed at low levels in breast cancer cells has been identified [54-56]. ERβ and ERα (called ER in this report) have a high conservation of amino acid sequence in hormone binding and DNA binding domains, but the structures of these proteins differ elsewhere.

The nuclear matrix has a central role in steroid hormone action [57-60]. ER is associated with the nuclear matrix of estrogen responsive tissues [4,5,61-63]. In *in vitro* reconstitution studies with nuclear matrices and hormone receptors (e.g., ER and androgen receptor), it has been

shown that nuclear acceptor sites for the hormone receptors are associated with the nuclear matrix [57,62,64]. The binding of the ER to the nuclear matrix was saturable, of high affinity, target tissue specific, and receptor specific [62]. Acceptor proteins for ER have been identified in a variety of estrogen-responsive tissues, including human breast cancer cell line MCF-7 [7,8,57,65,66]. In human breast cancer cells, some nuclear matrix acceptors for ER are ligand dependent. Protein acceptors for estradiol-ER differ from those for antiestrogen [4-(N,N-diethylaminoethoxy)4'-methoxy-α-(*p*-hydroxyphenol)α-ethyl-stilbene]-ER. Importantly, antiestrogen-resistant MCF-7 cells had a lower level of acceptors for antiestrogen-ER than did antiestrogen-sensitive MCF-7 cells [8].

D. Purpose

We propose that nuclear matrix acceptors for ER direct ER to specific sites in the nucleus, and that there exist nuclear matrix acceptors that bind ER in a ligand-dependent manner. An objective of this research is to isolate the nuclear matrix acceptors for ER in human breast cancer cells. To achieve this goal, we have designed an expression vector that directs the synthesis of GFP-ER fusion protein. An advantage of using GFP is that the location of the fusion protein (e.g., GFP-ER) can be seen in living cells. Importantly, we have shown that the attached GFP does not interfere with the transactivation function of ER, and that GFP-ER attaches to the nuclear matrix. We have constructed cell lines stably

expressing GFP-ER under control of the tetracycline-on system. The GFP-ER will provide the means to isolate the nuclear matrix acceptors for ER. The nuclear matrix acceptor proteins maybe useful diagnostic and prognostic markers. The function and mechanism of action of the ER depends on its' associated proteins and therefore identifying these proteins is a critical step in understanding these processes. Since it is now known that transcriptionally active chromatin is selectively bound to the nuclear matrix [15-19], those proteins associated with both the ER and the nuclear matrix are likely to be important in modulating ER activity.

MATERIALS AND METHODS

Plasmids and Cell Lines. pCI-nGFP-ER was derived from pCI-nGL1-C656G [76] and pSG5-HEGO (a gift from Pierre Chambon). pCI-nGL1-C656G DNA expresses rat glucocorticoid receptor (which was removed in subcloning and replaced with the estrogen receptor coding sequence from pSG5-HEGO), from the cytomegalovirus promoter/enhancer, and is tagged at the N-terminus with His_6, the hemagglutinin (HA) epitope, and green fluorescent protein (GFP) with the S65T mutation and humanized codon sequence. Cells lines used were MCF7, MDA MB 231, MDA MB 435A, MCF7(rTet) and MDA MB 435A(RTA-1). All cell lines were maintained in DMEM with 5% fetal bovine serum unless otherwise indicated. MCF7 (rTet) cells were a gift from Dr. R. Shui, and are stably transfected with the plasmid pUHD172-1neo that directs the expression of the reverse Tetracycline repressor and activating domain of VP16 protein and the neomycin resistance gene. MDA MB 435A (RTA-1) cells which are also stably transfected with pUHD172-1neo were a gift from Dr. Adrian Lee. The His_6-HA-GFP-ER coding sequence was subcloned into pUHD10-3, to place it under control a tetracycline inducible operon to create the plasmid pUHD10-3-GFP-ER. This plasmid was co-transfected with pCEP4 (contains hygromycin gene) into both the MCF7(rTet) and MDA MB 435 (RTA-1) cells. Transfected cells were grown in media containing Geneticin (Gibco BRL) and Hygromycin B (Calbiochem) to select for stable clones. The plasmids pERE-tk-CAT an ERE containing reporter plasmid [77], and pCH110 a β-galactosidase reporter plasmid

(Pharmacia Biotech) were used for transient transfection reporter assays.

Transient Transfections and ERE Reporter Assays. MB MDA 435 cells were plated in 100mm dishes in phenol red-free DMEM plus 5% twice charcoal stripped fetal bovine serum 2 days prior to transfection and were given fresh media 24 hours prior to transfection. Cells were maintained at 37°C in 5% CO_2 incubator prior to transfection. Cells were transfected by the $CaPO_4$ /BES precipitation method (Current Protocols in Molecular Biology, 9.1.7 Supplement 36). One ml of precipitate contained 4µg of pCH110 (β-galactosidase reporter plasmid) as an internal control, 5µg of ERE-tk-CAT reporter plasmid, pCI-nGFP-ER in amount indicated, and pCEP4 as carrier DNA to total of 15µg of DNA. Cells were in contact with precipitate for 14 hours and then washed twice with 1XPBS and fed fresh phenol red-free DMEM plus 5% twice charcoal stripped FBS containing 17β-estradiol, ICI 182 780 or 4-hydroxytamoxifen as indicated. Cells were harvested 30 hours after precipitate was removed. All transfections were done in triplicate. Chloramphenicol acetyltransferase (CAT) assays were done using the method in Current Protocols in Molecular Biology 9.7.5 supplement 29. β-galactosidase assays were done as described in Molecular Cloning (Maniatis) 16.66. CAT activity was normalized to β-galactosidase activity and the means and standard errors of the means of three determinations were plotted in figure 4.

Isolation of Nuclear Matrix. Protocol for isolation of nuclear matrix on coverslips or chamber slides was modified from He, Nickerson, and Penman [22]. Everything was done on ice unless otherwise indicated. Aspirate media from cells grown on acid washed coverslips in 35 mm wells or on chamber slides. Wash cells with ice cold PBS and aspirate. Add 2 ml cytoskeletal buffer (10 mM PIPES pH6.8, 300 mM sucrose, 100 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, pH to 6.8 with NaOH, then add 0.5% Triton X 100, 1mM PMSF). Incubate on ice 3-6 min. This step will remove soluble proteins, both cytoplasmic and nucleoplasmic. Aspirate off buffer. Add 2 ml Digestion buffer (10 mM PIPES pH6.8, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$ $6H_2O$, 1 mM EDTA, pH to 6.8 with NaOH, then add Triton X 100 to final concentration of .5%, 1mM PMSF and DNase I to final concentration of 168 u/ml). Incubate room temp, 30 min. Nuclei of cells are now much more pronounced under light microscope. This step will remove DNA and histones. Aspirate off buffer. Add 2 ml Extraction buffer (10 mM PIPES pH6.8, 250 mM ammonium sulfate, 300 mM sucrose, 3 mM $MgCl_2$, 7 mM EDTA, pH to 6.8 with NaOH, add Triton X 100 to .5%, PMSF to 1 mM). Incubate on ice 3-5 min. This step will remove remaining histones and strip the cytoskeleton except for intermediate filaments which remain tightly anchored to nuclear lamina. Aspirate off buffer. Add 2 ml 2M NaCl buffer (10 mM PIPES, 300 mM sucrose, 2 M NaCl, 3 mM $MgCl_2$, 1 mM EDTA, pH to 6.8 with NaOH, add PMSF to 1 mM final concentration). Incubate 3-5 min on ice. This step strips some proteins from the nuclear matrix and either uncovers or stabilizes the 10 nm filaments that form the core structure. Aspirate

buffer. Add 2 ml of PBS to cells on ice. Flip coverslips over onto slides (add drop PBS to keep moist) and view under fluorescent microscope.

Protocol for preparation of nuclear matrix in suspension was derived from reference 20-23 and outlined in figure 1.

Crosslinking of GFP-ER with Nuclear Matrix. NM2-IF (see figure 1) from cells transfected with pCI-nGFP-ER were crosslinked with DTBP (dimethyl 3,3'-dithiobispropionimidate-2HCl) (Pierce) according to the manufactures recommendations. Briefly the NM2-IF pellet was resuspended in .1M HEPES pH 8.5, so that protein concentration was 1µg/µl. DTPB was added to a final concentration of 10mM, and the sample was incubated at room temperature for 60 min. The sample was then denatured in SDS-loading buffer (without reducing agent) and boiled for 5 minutes prior to loading on SDS-PAGE gel.

Purification of GFP-ER using Metal Affinity Resin. Talon metal affinity resin from Clonetech was used according to the manufactures directions for purification of GFP-ER.

RESULTS AND DISCUSSION

A. Construction of ER Fusion Protein

In collaboration with Dr. Gordon Hager and Dr. Han Htun, we have constructed a vector pCI-nGFP-ER, that when transiently transfected in human breast cancer cell lines directs the expression of an ER fusion protein we call GFP-ER (Fig. 3). This ER fusion protein (GFP-ER) has three N-terminal tags that simplify its detection and isolation. The first, is the His_6 tag which facilitates the isolation of the fusion protein using metal chelating columns or beads. Second, is an HA epitope tag which can be detected on western blots with anti-HA antibodies. Third, is the Green Fluorescent Protein (GFP) which makes it possible to observe GFP-ER in living cells using fluorescent and confocal microscopy.

B. GFP-ER IS TRANSCRIPTIONALLY COMPETENT

To determine if the GFP-ER was a functional protein, transient transfection assays were preformed in the ER negative MDA MB 435A breast cancer cell line using the ERE-tk-CAT reporter vector. As expected a dose dependent effect was observed with 17β-estradiol, with maximum induction observed at 10nM (Figure 4A, lanes 1-5). Both the anti-estrogens ICI 182 780 and 4-hydroxytamoxifen did not induce the ERE-tk-CAT plasmid and ICI 182 780 had an inhibitory effects (Figure 4A, lanes 6 and 7). When 500nM ICI 182 780 was added in the presence of 10nM 17β-estradiol it completely inhibited the induction of ERE-tk-CAT by estradiol, as would be expected from a normally functioning ER (Figure

4A, lane 8). Likewise, when 250nM 4-hydroxytamoxifen was added in the presence of 10nM 17β-estradiol, it also inhibited the induction of ERE-tk-CAT by estradiol in the expected manner (Figure 4A, lane 9). Also, as typical of ER transfection reporter assays, an increase in CAT activity was observed with greater amounts of GFP-ER expression plasmid transfected, while the relative degree of induction with 17β-estradiol decreased with increasing amounts of GFP-ER expression plasmid effect (Figure 4B). Together this data shows that the GFP-ER fusion protein has ligand depended transactivation activity, and functions in a manner consistent with a wild type estrogen receptor.

C. Nuclear Localization of GFP-ER

Because of the GFP tag, the GFP-ER fusion protein could be seen in living cells using fluorescent microscopy. In MCF-7 (ER+) and MDA MB 231 (ER-) human breast cancer cells cultured in either the presence or absence of 17β-estradiol, transiently expressed GFP-ER protein was found only in the cells nucleus with exclusion from the nucleoli (Fig. 5). In the transiently transfected MCF-7 cells cultured in phenol red free media with 5% twice charcoal stripped fetal bovine serum (i.e. no added ligand), a punctate pattern of nuclear localization was seen, while in MDA MB 231 cells the pattern was more diffuse (Figure 5A). When 17β-estradiol was added to the media, the GFP-ER pattern in MCF-7 cells remained the same as when no ligand was added, while in MDA MB 231 cells the pattern became more punctate or speckled (Figure 5B). The same effects

were observed when 4-hydroxytamoxifen was added to the media. These results indicate that the presence of ligand and cell type effect the nuclear distribution of the GFP-ER; this may be due to alterations in affinity for nuclear matrix. However, further experiments must be done before any conclusions can be drawn.

D. GFP-ER Localizes to the Nuclear Matrix

Nuclear matrices were isolated on chamber slides from MCF-7 and MDA MB 231 cells expressing GFP-ER by digesting with DNAase I and then extracting with 0.25 M ammonium sulfate and 2 M NaCl. Fluorescent and confocal microscopy analysis of these nuclear matrices showed that GFP-ER remained associated with the nuclear matrix of both MCF-7 and MDA MB 231 cells (Fig. 6). Further, the nuclear localization of GFP-ER observed in these cells was preserved in nuclear matrices. These observations suggest that nuclear matrix acceptors guide the ER to specific nuclear sites. Nuclear matrices were also prepared from a suspension of MCF-7 cells using the protocol shown in Fig. 1. To isolate nuclear matrices, nuclei isolated in isotonic conditions are digested with DNAase I at room temperature and then extracted with 0.25 M ammonium sulfate, yielding NM1-IF (nuclear matrix with associated intermediate filaments) [6,31] (Fig. 1). The intermediate salt extraction protocol reduces or eliminates the precipitation that may occur with a subset of the nuclear proteins when high salt is used directly to extract non matrix nuclear protein [22]. The NM1-IF nuclear matrices are further extracted

with 2 M NaCl, yielding NM2-IF. Western blot analysis of proteins in the various fractions with an anti-HA antibody showed that GFP-ER was associated with the nuclear matrix (Fig. 7).

E. Purification of GFP-ER.

In preliminary studies, we have shown isolation GFP-ER from nuclear matrices of MCF-7 cells expressing GFP-ER using the Talon metal affinity resin was possible (Fig. 7). Metal affinity resins can be used to isolate proteins with His_6 tags. In future, we plan to modify this protocol to further purify the GFP-ER by additionally doing immunoprecipitation with anti-HA antibodies and protein A [68]. This protocol will also be used to purify GFP-ER crosslinked proteins.

F. GFP-ER Crosslinks to Proteins in the Nuclear Matrix

Nuclear matrices isolated from MCF-7 cells expressing GFP-ER were treated with DTBP (dimethyl 3,3'-dithiobispropionimidate-2HCl), a reversible crosslinker, and then nuclear matrices were dissolved in a SDS buffer. Western blot analysis of the nuclear matrix proteins with anti-HA antibody to detect GFP-ER showed that GFP-ER was present in several bands migrating slower than GFP-ER on SDS gels (Fig. 7). This study shows that crosslinking GFP-ER to nuclear matrix proteins is feasible. In the future, the crosslink between GFP-ER and the bound protein will be reversed by the addition of a reducing agent (e.g., β-mercaptoethanol). The proteins bound to GFP-ER will be analysed by one-dimension SDS

gel and two-dimension gel electrophoresis [69,70], and in Western blot experiments [71].

There are several candidate nuclear matrix proteins that may bind to ER in hormone dependent breast cancer cells. There is direct and indirect evidence that ER binds to several proteins, including GATA-1, Sp1, SRC-1, CBP/p300, ERAP, RIP140, TFIIB, SWI2/SNF2, cyclin D1; many of these proteins interact with ER in a ligand dependent manner [43,50,72-74,75]. Some of these proteins are known to be associated with the nuclear matrix. For example, Sp1 is a nuclear matrix associated transcription factor that may bind to GFP-ER [25,50]. Antibodies to several of these proteins interacting with ER are commercially available, and in Western blot experiments we will find whether the protein is associated with nuclear matrix bound GFP-ER.

The rationale for crosslinking GFP-ER while attached to the nuclear matrix rather than at an earlier stage in the fractionation procedure is to avoid crosslinking of ER to non-matrix nuclear proteins. Although our goal is to identify nuclear matrix proteins interacting with ER, it will be of interest to apply the DTBP reagent to intact nuclei isolated from cells expressing GFP-ER. Following isolation of GFP-ER crosslinked proteins, we will analyse these proteins bound to GFP-ER on two dimension gel patterns. This gel pattern will be compared to that containing proteins crosslinked to GFP-ER on nuclear matrices.

The ultimate goal of these studies is to identify nuclear matrix acceptor proteins for the ER-estradiol and ER-antiestrogen. We expect

that human breast cancer cells that are ER+ but differ in hormone responsiveness and/or resistance to antiestrogens will exhibit a different spectrum of the nuclear matrix ER acceptor proteins [8].

G. MCF7 AND MDA MB 435A STABLE CELL LINES

To isolate nuclear matrix acceptors for ER (± ligand), I will use MCF-7 (ER+, hormone dependent) human breast cancer cell lines stably transfected with the DNA construct directing the expression of GFP-ER. We have chosen to regulate the expression of GFP-ER by introducing the tetracycline-on system [67] into these cells (Fig. 8). MCF-7(rTet) and MDA MB 435A (RTA-1) are cell lines that have been stably transfected with the plasmid pUHD172-1neo that directs the expression reverse tetracycline-controlled transactivator (rtTA, a fusion between reverse Tetracycline repressor and activating domain of VP16 protein) and neomycin resistance gene (Fig. 8). GFP-ER was cloned into pUHD10-3, placing GFP-ER under the control of a tetracycline inducible operon. In the absence of doxycycline, rtTA does not activate the expression of GFP-ER, but when doxycycline is added, there is doxycycline concentration dependent activation of GFP-ER expression. We have selected several stable MCF7(rTet) GFP-ER clones, and are in the process of selecting MDA MB 435A (RTA-1) GFP-ER stable clones. Having stable GFP-ER cells lines will make isloation and characterization of GFP-ER crosslinked proteins more efficient than using transiently transfected cells.

SUMMARY OF PROGRESS IN RELATION TO STATEMENT OF WORK

The efforts for the first year according to the Statement of Work in the original proposal were to be focused on specific aims 1 and 2.

Specific Aim 1: To determine how the nuclear matrix affects the activity of the ER. In my original proposal the plan to address this question was to use *in vitro* transcription assays done with a reporter construct which contained estrogen receptor elements (EREs) for ER binding. These assays were to be done in the presence and absence of nuclear matrices from both ER+ and ER- breast cancer cell lines. If ER bound to the nuclear matrix was active then, *in vitro* transcription assays done with the addition of nuclear matrices from ER+ breast cancer cell lines plus estrogen, were expected to have elevated levels of expression of the reporter gene. If the nuclear matrix affected the activity the ER then, *in vitro* transcription assays done with purified ER (prepared by *in vitro translation*) +/- estrogen, were proposed to have different levels of activity depending on the addition of nuclear matrix from breast cancer cells which are ER negative.

I have significantly modified my approach to this specific aim for the following reasons. The last several years have seen a growth in understanding of the importance of associated proteins for ER function. My own experience with ER electromobility gel shift assays found great differences in activity depending on how the ER was prepared and isolated [75]. Therefore, it has become clear to me that the experiments as originally proposed are likely to be difficult to perform and even more

difficult to interpret! Comments from Grant Reviewer A (from the US Army review of the original proposal) about these experiments that "*In vitro* estrogen-dependent transcription has been notoriously difficult to achieve. Consequently, it is an interesting idea to test the effect of the nuclear matrix. However, if the matrix plays a structural role in the transcription of these genes, it is unlikely that matrix fragments added *in vitro* will have the same effect." Have further deterred me from pursuing these particular experiments.

My current hypothesis on the role of the nuclear matrix in affecting estrogen receptor activity is that the matrix provides a solid support on which to organise the ER and its associate proteins which are responsible for modulating its' activity, and that the associated proteins will change depending on ligand and the stage of cancer and of course whether the ER is associated with the matrix. Therefore, the identification of the ER associated proteins when the ER is associated with the matrix is key to understanding how the nuclear matrix affects the activity of the ER. And this is exactly what is being done in specific aim 2.

Specific Aim 2: To identify and isolate nuclear matrix acceptor proteins for the ER. While some of the details of how I planned to accomplish specific aim 2 have been modified, the primary goals for year one of constructing and epitope tagged ER, checking to see if it was still a functional ER and preparing stable cell lines expressing the epitope tagged ER have all been accomplished. In addition I have been able to

look at the nuclear localization and nuclear matrix association of the GFP-ER using the GFP tag. Also, I have preliminary data demonstrating that I will be able to purify and isolated the GFP-ER and its' associated proteins. In the next year I plan to focus on identifying nuclear matrix proteins associated with ER, as all of tools to achieve this goal are now in place. This will take priority over specific aims 3 and 4 which are to determine if *c-myc* and other DNA sequences are associated with ER-nuclear matrix complexes and to determine if ectopic MAR sequences of transgenes associate with the nuclear matrix.

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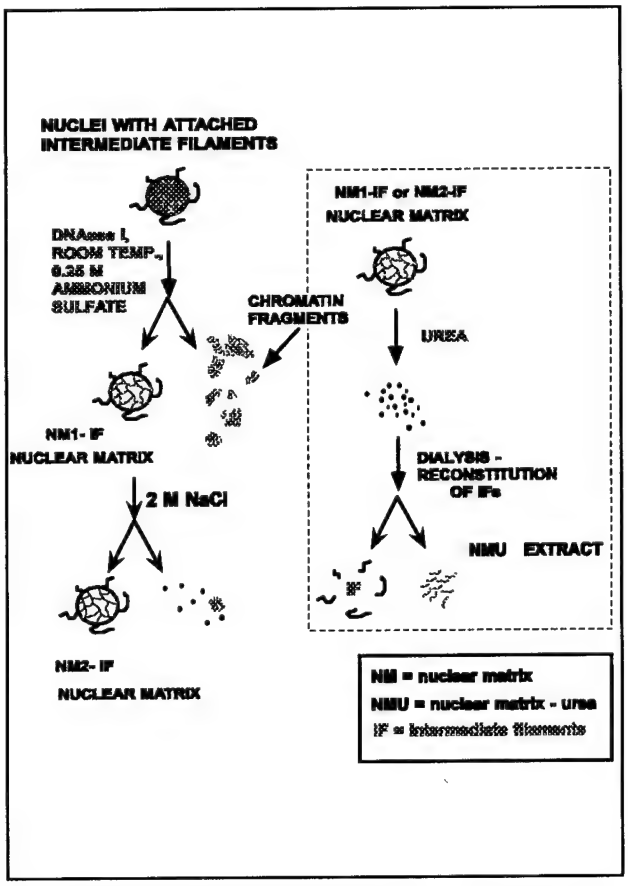


Figure 1. Isolation of nuclear matrix proteins. NMU contains nuclear matrix proteins.

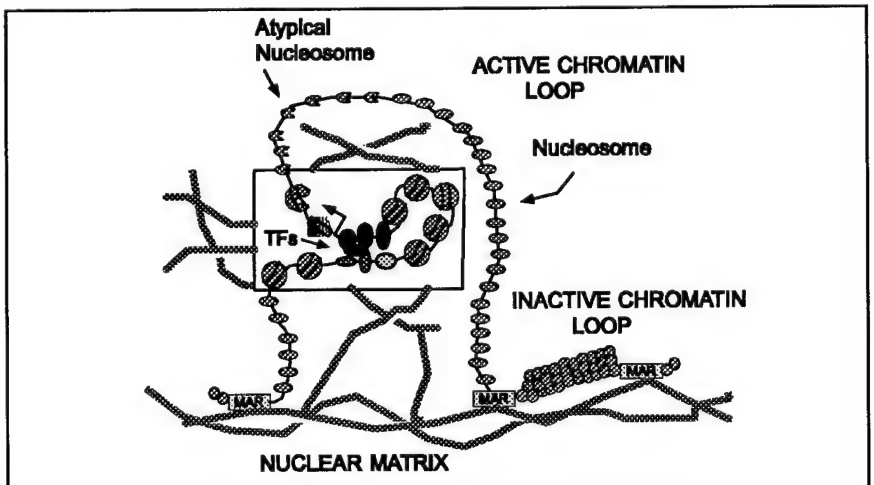


Figure 2. Organization of nuclear DNA (TFs = transcription factors, some of which are associated with nuclear matrix)

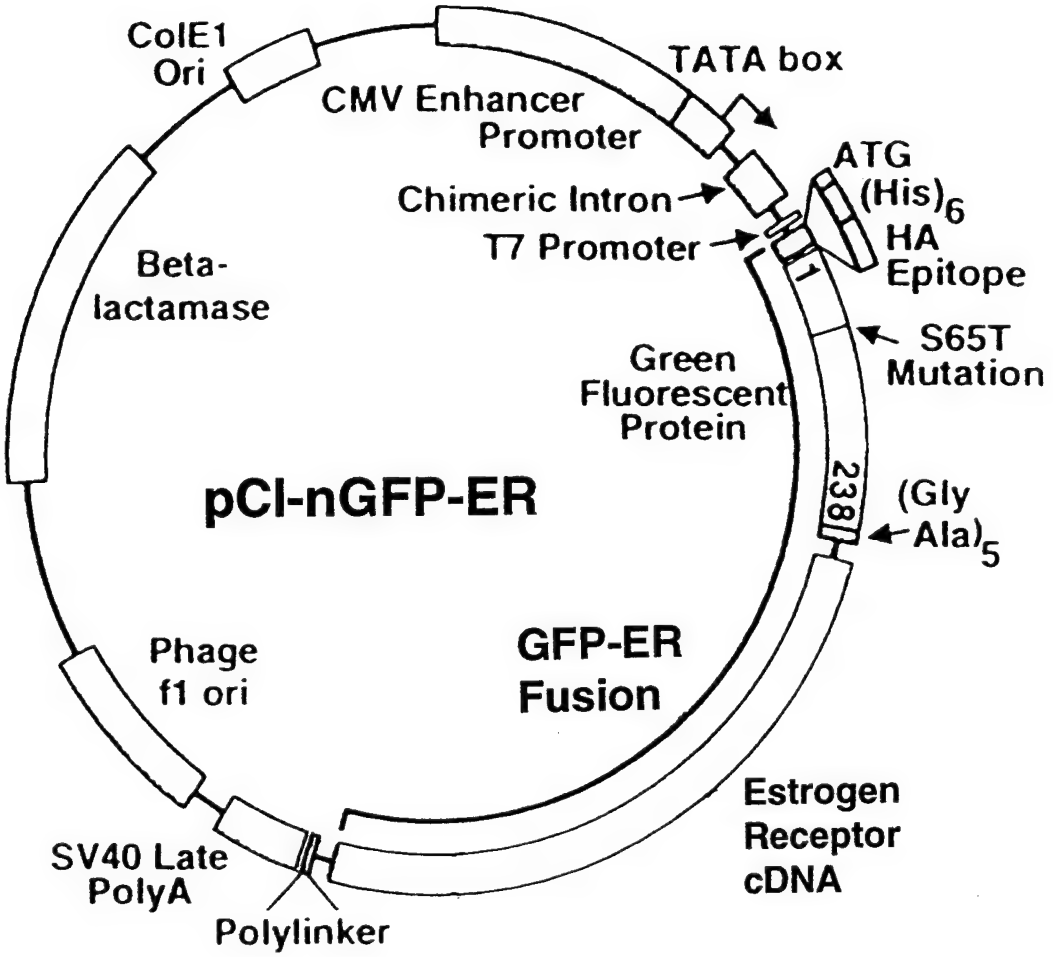


Figure 3. pCI-nGFP-ER expression vector. This plasmid expresses an estrogen receptor fusion protein which has a His_6 tag, a HA epitope tag and the green fluorescent protein (GFP). The expressed protein is called GFP-ER.

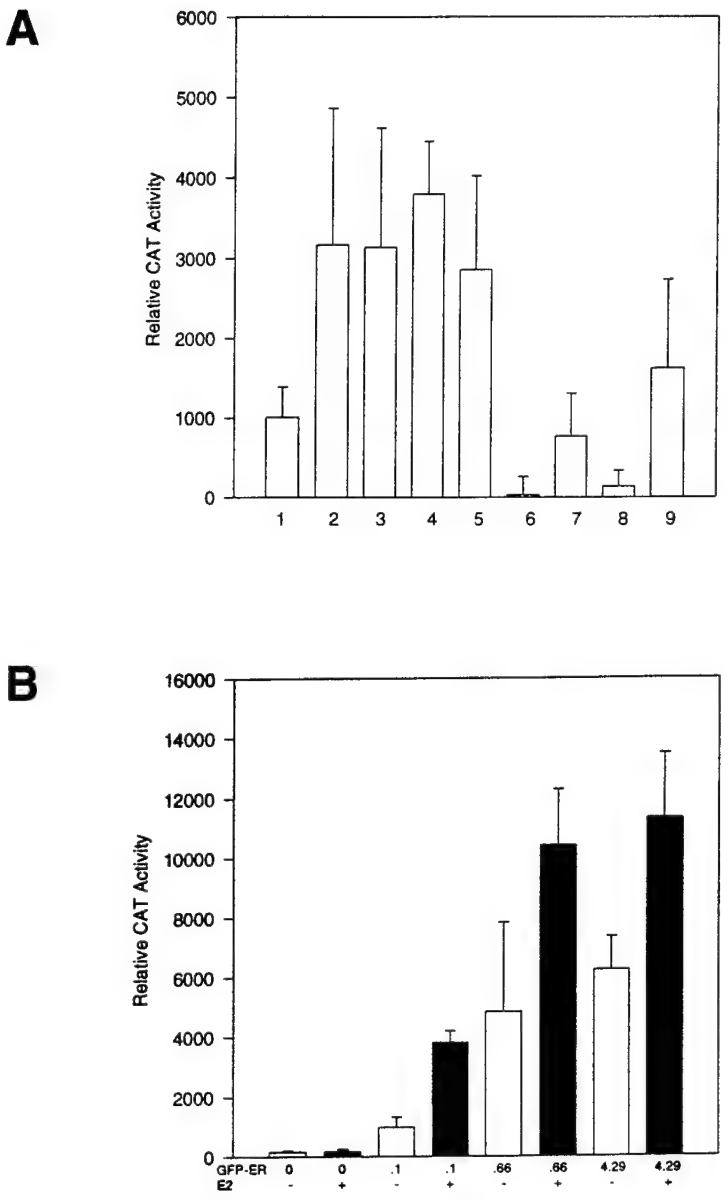


Figure 4. Ligand Regulation of the pERE-tk-CAT reporter vector by GFP-ER in MDA MB 435A cells. Panel A. Lane 1 - no ligand added. Lane 2 - 0.1nM 17β-estradiol. Lane 3 - 1nM 17β-estradiol. Lane 4 - 10nM 17β-estradiol. Lane 5 - 100nM 17β-estradiol. Lane 6 - 10nM ICI 182 780. Lane 7 - 10nM 4-hydroxytamoxifen. Lane 8 - 500nM ICI 182 780 and 10nM 17β-estradiol. Lane 9 - 250nM 4-hydroxytamoxifen and 10nM 17β-estradiol. (All transfections in Panel A are with 0.1μg of pCI-nGFP-ER). **Panel B**. White bars represent no ligand added, black bars represent 10nM 17β-estradiol added. Increasing amounts of GFP-ER expression plasmid (μg) were transfected. All experiments done in triplicate.

A

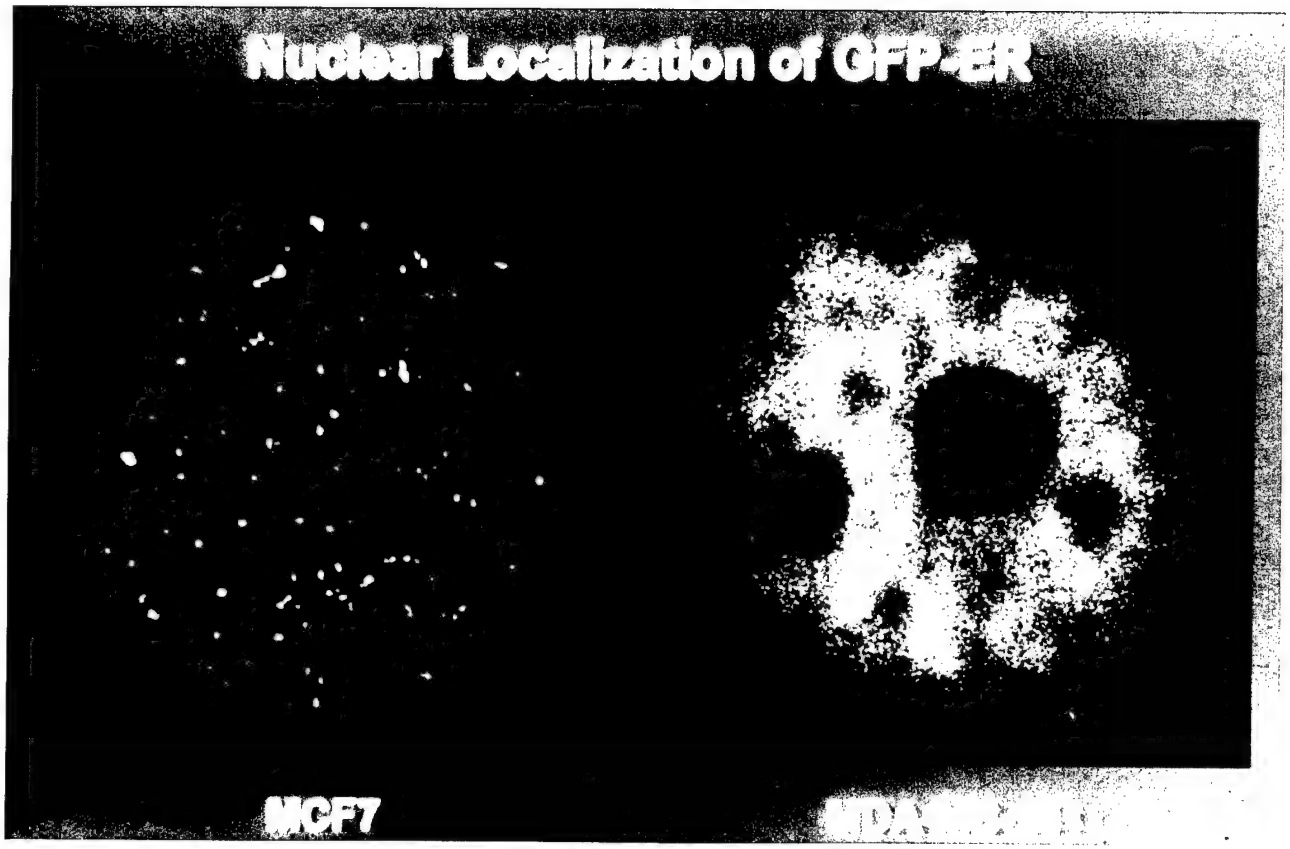


Figure 5A (legend on following page)

B

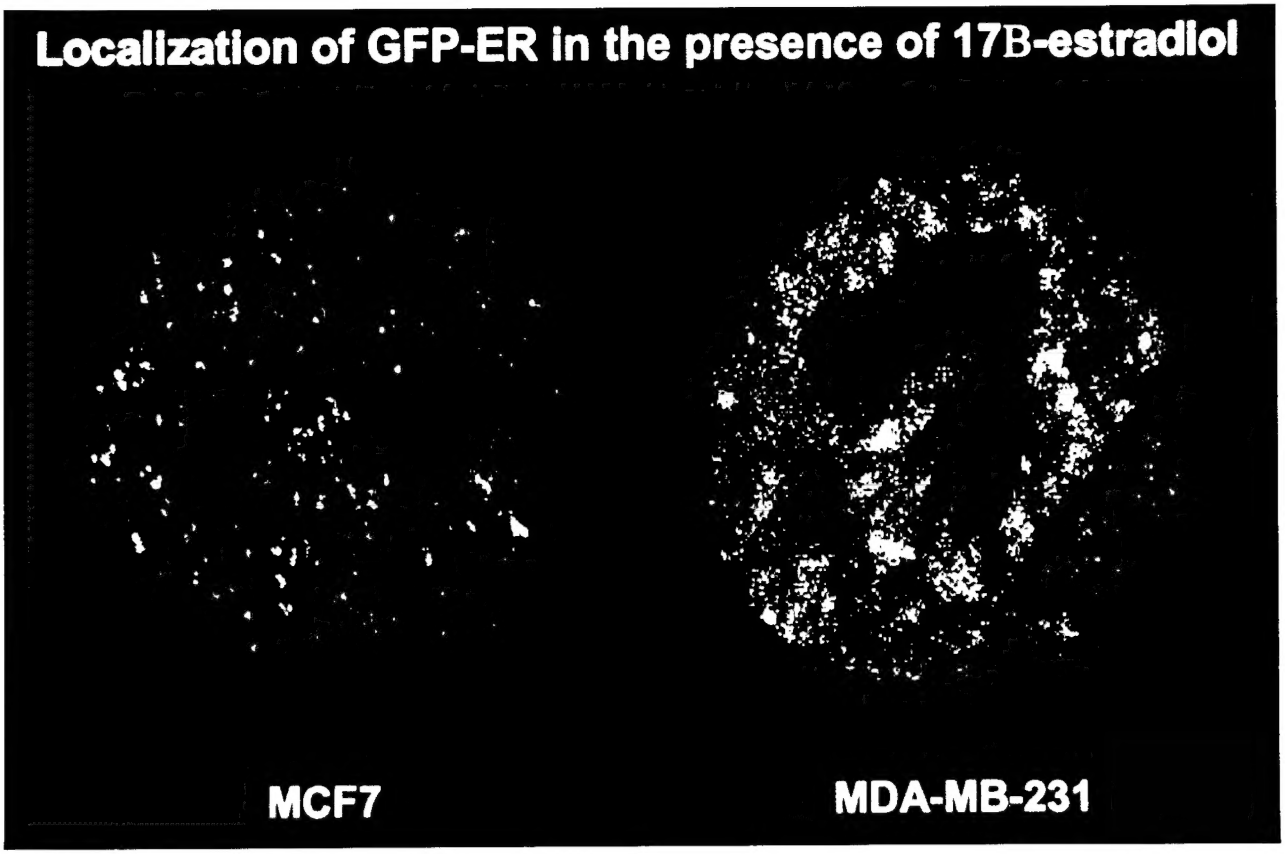


Figure 5. Confocal images (depth of field 0.5μ) of the nuclei of MCF7(ER+) and MDA MB 231(ER-) human breast cancer cell lines, showing the nuclear localization and distribution pattern of GFP-ER. Panel A - cells grown in phenol red free media with 5% charcoal striped serum. **Panel B** - cells grown under same conditions as in panel A, but with 10nM 17β-estradiol added.

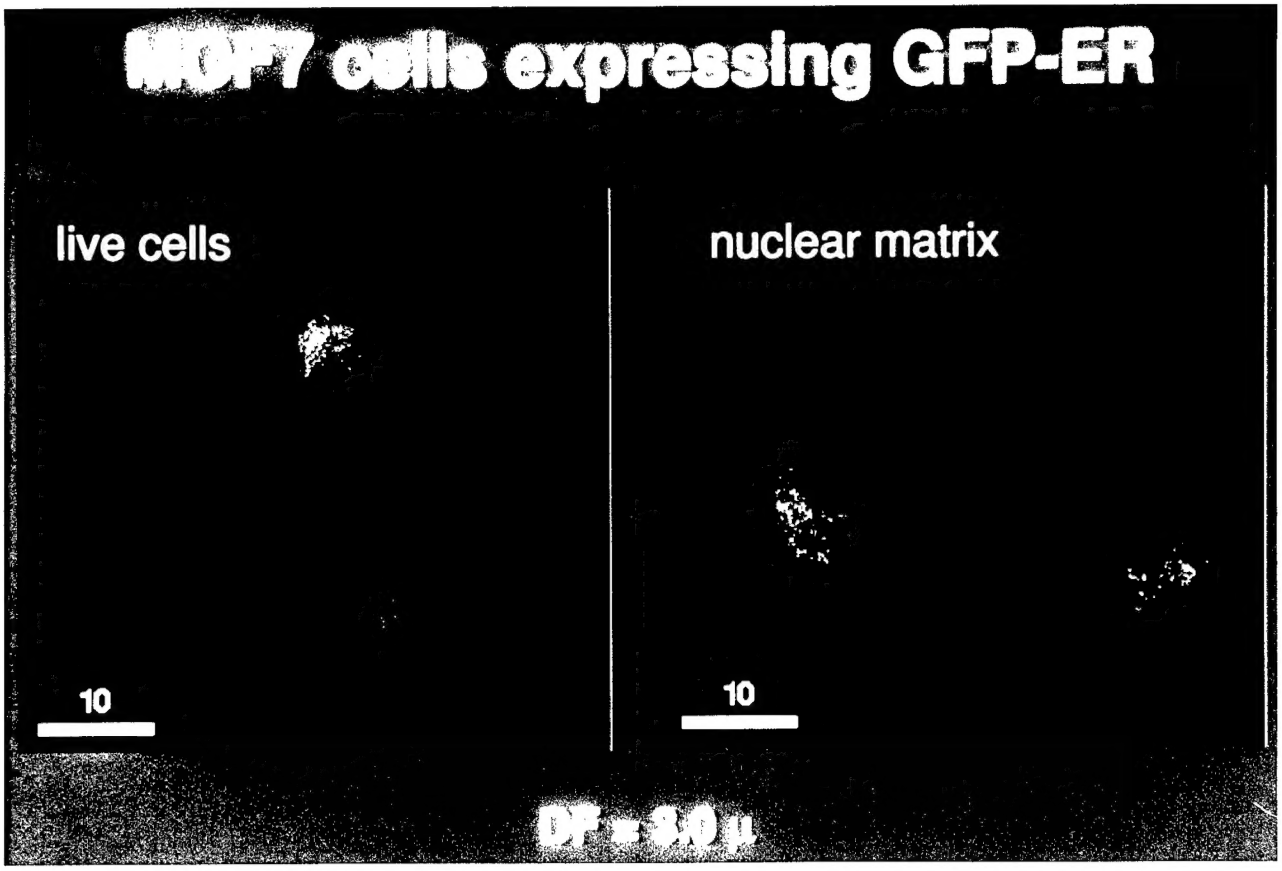
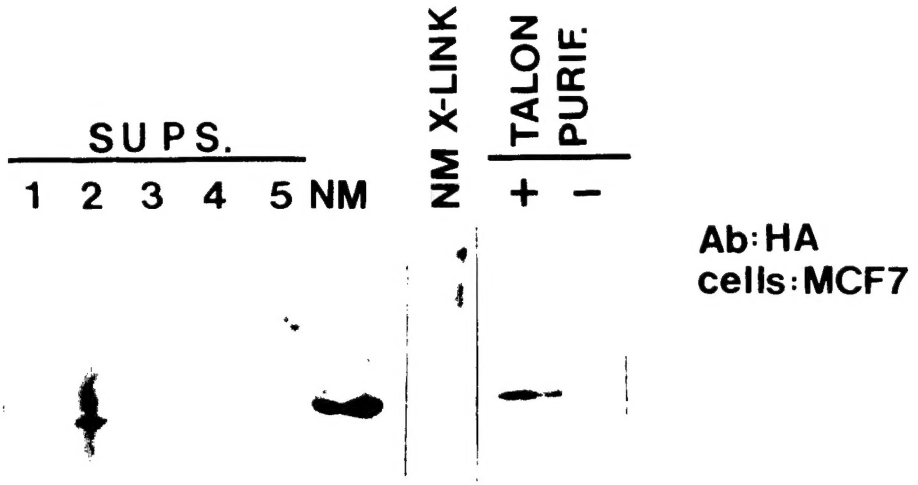


Figure 6. Confocal images (depth of field 3.0μ) of living MCF7 cells and nuclear matrices from MCF7 cells isolated on cover slips.



FXN.	PORTION FXN.	EXTRACTION
1	0.1	TNM+0.5% TRITON X-100
2	0.1	TNM
3	0.25	DNase I → 0.25M $(NH_4)_2SO_4$
4	1.0	2M NaCl
5	1.0	2M NaCl
NM	1.0	

Figure 7. Biochemical analysis of GFP-ER localization and purification. Western blots of SDS-PAGE gels with the anti-HA antibody (detects GFP-ER which has a HA-tag). The first six lanes are all of the supernatants form a NM_2-IF isolation. GFP-ER seen in lanes 1 and 2 is leached from nucleus of cells as no GFP-ER is localized to cytoplasm in confocal images. Lane marked NM X-LINK is nuclear matrix which was crosslinked with DTBP, and then solubilized prior to running on SDS-PAGE gel. Lanes marked TALON PURIF. show product of Talon purification from cells expressing GFP-ER (+) or not expressing GFP-ER (-).

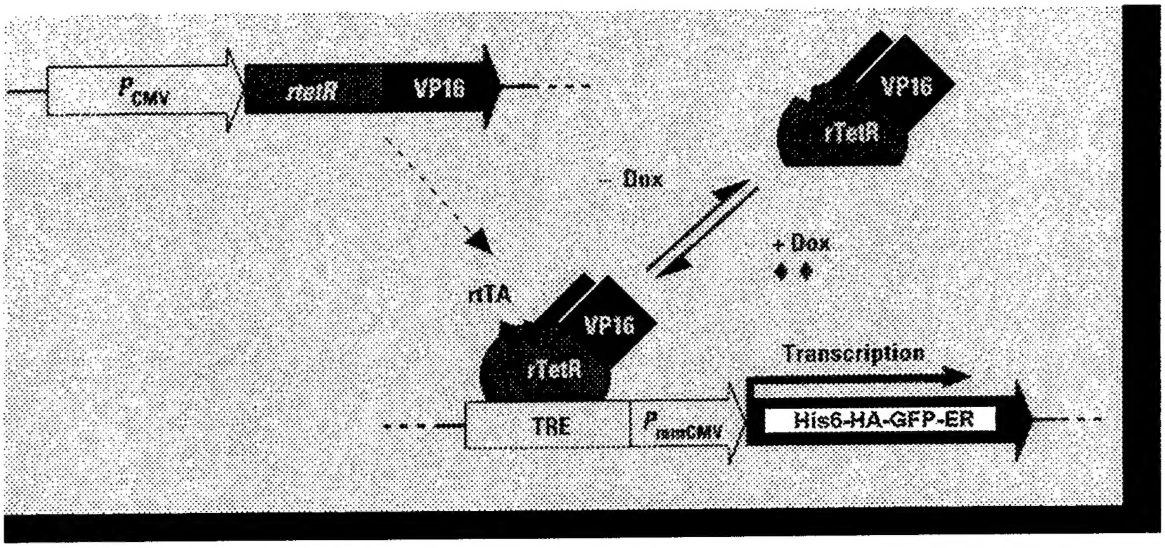


Figure 8. The tetracycline-on system.